Biomedical Reviews 1997; 8: 37-54

ISSN1310-392X

LOCAL EVENTS WITHIN THE INJURED AND REGENERATING PERIPHERAL NERVE TRUNK: THE ROLE OF THE MICROENVIRONMENT AND MICROCIRCULATION

Douglas W. Zochodne
Department of Clinical Neurosciences and The Neuroscience Research Group, University of Calgary, Calgary, Alberta, Canada

SUMMARY

• *Peripheral nerves respond to injury in a unique fashion. Changes in the local milieu of the injured nerve trunk may determine both the likelihoods of regeneration and the production of neuropathic pain. For example, changes in local blood flow within this microenvironment may reflect several interesting features of the repair process. Crushed and sectioned nerves develop hyperemia, or rises in local blood flow rather than ischemia, and these rises appear to be mediated by one of several mechanisms. Firstly, vasa nervorum, the blood vessels that supply nerve trunks, are innervated by peptidergic fibers that may participate in "neurogenic" inflammation, as occurs in other innervated tissues. Secondly, following a nerve section or crush, early rises in blood flow may be mediated by local deposition of calcitonin gene-related peptide and nitric oxide from axonal endbulbs. Thirdly, brisk angiogenesis accompanies a proliferative phase in the proximal nerve stump that accompanies mast cell proliferation and axonal sprouting. Axonal sprouting, in turn, may be supported by local trophic factors, and the success of subsequent regrowth down the distal nerve stump may be determined by the microenvironment it encounters on its road to recovery. Better understanding of these and other events in injured nerve trunks is needed to help solve the two cardinal problems of peripheral nerve injuries: functional disability from impaired regeneration and the development of disabling neuropathic pain. (**Biomed Rev 1997; 8:** 37-54)*

Received for publication 28 October 1997 and accepted 30 November 1997.
Corespondence and reprint requests to DrD.W. Zochodne. Department of Clinical Neurosciences, University of Calgary, Room 182A, 3330 Hospital Drive, NW, Calgary, Alberta, Canada T2N4N I.Tel: 1 (403) 2208759,Fax: 1 (403)2838731,E-mail:dzochodn@acs.ucalgary.ca

INTRODUCTION

• Peripheral nerve damage arising from trauma or dis ease (acquired or inherited neuropathies) is common and dis abling. Our understanding of local repair mechanisms of in jured peripheral nerves, however, is incomplete. The attempted regeneration of fibers occurs within a nerve trunk microen vironment milieu replete with inflammatory mediators, trophins, microvascular changes and proliferating cellular elements. How this milieu develops and influences regenera tion is uncertain. This contrasts with the certainty that recov ery from human axonal degeneration is slow, often incomplete and frequently painful.

Why is the microenvironment important? The classical monograph written in 1949 by William Lyons and Barnes Woodhall entitled "Atlas of peripheral nerve injuries" (1) catalogued pathological changes of peripheral nerve specimens taken by surgeons attempting to repair war wounds of American soldiers in World War II. The procedures that provided these specimens were attempts, sometimes futile, to

relieve obvious disability and pain by surgical approaches. The micrographs are dramatic, with examples of neuromas (a swollen mass of ineffectively regenerating peripheral nerve sprouts), neuromas in continuity, stretch and compression injuries, inadequate attempts at repair, and other problems. There are photomicrographs of poorly regenerated specimens taken sometimes months after the injury with axons ineffectually attempting to penetrate masses of fibrotic tissue. Local microvessels are frequently thrombosed and tissue ischemic. A subsequent paper concentrates on these devastating microvascular changes (2).

Several of the themes in the story of peripheral nerve repair are beginning to emerge. It is clear that recovery from injury and the marshalling of regenerative processes follow an interesting and apparently coordinated timetable of actions that begin soon after the injury. Although likely to share features observed in any form of tissue repair, the peripheral nerve trunk recovers from injury in several unique ways. Axons must bridge injured gaps, and regrow through previously denervated nerve trunks to their targets. To do so, they must grow new axonal sprouts, penetrate the injury milieu and find their way into and down the distal nerve trunk. At each step of this process, the microenvironment must favour successful regeneration by delivering or generating trophic signals, by supplying metabolic requirements, and by maintaining a distal nerve trunk that is suitable to support large numbers of regrowing axons. Each of these steps involves, and is reliant upon the nerve trunk microcirculation, or vasa nervorum.

THE MICROCIRCULATION OF PERIPHERAL NERVES AND GANGLIA

• Anatomy of vasa nervorum

Peripheral nerve trunks have an intrinsic nutritive microcirculation, largely capillary, that is supplied by local and remote feeding arterioles from an extrinsic epineurial plexus (3). Endoneurial capillaries are large, nonfenestrated, with a prominent basement membrane, a near complete pericyte investment and frequent pinocytotic vesicles. Arterioles are often thin walled with a rudimentary internal elastic lamina making them sometime difficult to distinguish from venules. In the extrinsic epineurial plexus, arterioles (and perhaps venules) are innervated by vasoconstrictive adrenergic fibers, and vasodilatory peptidergic fibers containing substance P (SP) and calcitonin gene-related peptide (CGRP) (4-11). Nervi nervorum, or the fibers that innervate vasa nervorum, appear to be particularly expressed in the epineurium, but not in endoneurial microvessels (12). Some noradrenergic perivascular fibers also contain neuropeptide Y. Other epineurial perivascular fibers are immunoreactive to SP and CGRP or to CGRP alone. Axons immunoreactive to vasoactive intestinal peptide (VIP) innervate epineurial microvessels of the facial and vagus, but not sciatic nerves (12). An important characteristic of the vascular supply of peripheral nerves is their rich anastomotic blood supply (13,14). This rich arteriovenous (AV) shunt flow is particularly prominent in the epineurial plexus but both the epineurial and endoneurial compartments may be thought of as a large continuous interconnected vascular bed (3). Since the endoneurium contains relatively few arterioles, it is likely that nutritive flow in this compartment is regulated by local penetrating branches from or distant longitudinal branches from the epineurium, where vessel caliber is regulated.

• Local measurement of blood flow

Several methods are available for the measurement of local blood flow in small tissues like peripheral nerve and ganglia, but each offer advantages and disadvantages. Indeed some controversy has arisen over these techniques because of discrepancies in reports of changes of nerve blood flow in experimental diabetes. The topic of nerve blood flow measurement has been reviewed in detail elsewhere (15,16). Hydrogen clearance polarography measurements employ small (3-5 micron tipped) hydrogen sensitive microelectrodes that can be inserted within the endoneurium or dorsal root ganglia with minimal trauma. The clearance of hydrogen administered to saturation through a ventilatory gas mixture in paralysed and ventilated animals is measured by the microelectrode. Blood flow is calculated from a biexponential or monoexponential curve fit. Theoretical aspects of hydrogen clearance in general, and its application to nerve in particular, have been discussed (16,17). The slow component of biexponential curves correlates with endoneurial blood flow (EBF), as measured by other techniques, whereas the fast component has contributions from the epineural plexus and AV shunts. While the hydrogen clearance polarography technique is somewhat invasive and requires 30-60 minutes of data collection time, it offers quantitative data selective for the endoneurial space and permits serial blood flow studies to be made in the same preparation, for example, before and after pharmacological intervention. The theoretical sphere of sensitivity for hydrogen microelectrodes has been reported to be approximately 30 microns (18,19).

The laser doppler flowmetry (LDF) technique measures red blood cell (RJBC) flux in the tissue sampled beneath afferent and efferent fiberoptic probes. The signal recorded is a function of the laser signal to the afferent probe reflected from moving RBC, and its magnitude depends on the velocity and number of moving RJBC. LDF is simple to apply but there are important caveats toward obtaining reproducible signals. The recordings, influenced by ambient room light including heating lamps, are dependent on probe position, and individual

measurements can be quite variable along the length of a peripheral nerve because of variations in the anatomy of the epineurial vascular plexus. Measurements should be made under strict conditions with all light sources turned off and a mean RBC flux signal should be calculated from pooled multiple individual measurements along the nerve trunk (20). Unlike hydrogen clearance polarography, the signal obtained with LDF is a relative flow signal. By taking measurements over the epineurial surface of the nerve trunk, the signal is biased toward measurements of epineurial blood flow, despite limited penetration and sampling of the endoneurium. The technique provides real time data and does not require animal sacrifice. Other measurement techniques employed to measure peripheral nerve perfusion include tracer methods using radiolabelled microspheres, or labelled iodoantipyrene (21). These techniques provide only single measurements. AV shunting of microspheres may give inappropriately low values of EBF. Video angiology allows direct visualization of epineurial vessels and India-ink perfusion is useful in outlining microvessels for morphometric studies (9,22).

- **Nerve blood flow**

In resting anaesthetized rats, normal EBF has been measured between 15 and 20 ml/lOOg/min, [19.1±0.4; n=161; (23)] using hydrogen clearance polarography with the nerve bathed in a pool of mineral oil at 37°C. Microvascular resistance is calculated as MAP/EBF (6.93±0.18 mmHg. 100g.min.ml-l; n=161), where MAP is mean arterial pressure. Selective EBF measurements using hydrogen clearance polarography and labelled iodoantipyrene uptake have given similar values. Blood flow in the epineurial space has been more difficult to measure because of the presence of AV shunts. Microsphere measurements have suggested that blood flow is higher than that of the endoneurium, estimated as 33 ml/lOOg/min (21). By weighing contributions of the biexponential hydrogen clearance curves, a "composite" blood flow measurement, taking into account endoneurial, epineurial, and AV shunt flow was estimated as 32.0±1.2 ml/lOOg/min; n=161 a value similar to iodoantipyrene results (27±3 ml/lOOg/min; n=14 (21).

Under normal physiological conditions, there appears to be both tonic adrenergic and peptidergic tone on vasa nervorum, and overall changes in nerve blood flow follow those of MAP passively, indicating little autoregulation (24,25). EBF increases and decreases in a curvilinear fashion with changes in mean arterial pressure. EBF appears to be independent of CO_2, whereas epineurial flow gauged real time by LDF had some limited CO_2 sensitivity (26). The short term administration of an a-adrenergic blocking agent, such as phentol-amine, or chronic sympathectomy by guanethidine is associated with rises in EBF (8,9). Live video microscopy of epineurial vessels exposed to norepinephrine identified an interesting segmental pattern of vasoconstriction suggesting an uneven distribution of alpha receptors along the vessel, perhaps indicating that there are specific zones of receptor clustering, where feeding vessels control downstream perfusion (9) (Fig. 1). Vasa nervorum are also sensitive to vasoconstriction from endothelin-1 (ET-1), a peptide with yet greater potency than that of norepinephrine. In experiments with topical application of endothelin over epineurial vessels, ET-1 induced endoneurial ischemia that resulted in transient acute conduction block of myelinated motor fibers (27). In diabetics, ET-1 induced endoneurial ischemia caused frank nerve infarction (28).

Administration of specific antagonists of either SP or CGRP, is associated with vasoconstriction and a fall in EBF (29). Thus, vasa nervorum have alpha receptors that mediate vasoconstriction in response to exogenous norepinephrine and CGRP receptors, likely type-1 (30), that cause vasodilatation when exposed to exogenous peptide. CGRP is a highly potent vasodilator, with actions mediated in part by ATP-sensitive K^+ channels (following activation of adenyl cyclase and generation of local cyclic AMP), but also in part related to cyclic GMP and nitric oxide (NO) produced by endothelialNO synthase (eNOS) (31-34). While tonic CGRP vasodilation may operate through eNOS in some vascular beds, it may be that vasa nervorum dilatation by CGRP is NO independent, instead acting wholly through ATP-sensitive K^+ channels. Inhibition of NOS had no influence on resting nerve blood flow (35). The role of SP receptors is unclear: CGRP is a much more potent and longer acting vasodilator than SP (10). Selected prostaglandins have also been demonstrated to dilate vasa nervorum: PGE,, PGL, and PGF_2 (36).

- **Ganglia blood flow**

Local blood flow in sensory and autonomic ganglia has not been measured as frequently as in nerves. Using hydrogen clearance microelectrode polarography, L4/5 dorsal root ganglia blood flow measured 36.1±2.7 ml/lOOg/min, (n=13), or approximately twice the value for nerve (37). Unlike peripheral nerves, we did not detect a relationship between MAP and ganglion blood flow until MAP fell below 60 mmHg. The absence of a relationship indicated that ganglia, unlike nerves, possessed some degree of autoregulation. Hypercarbia and hypocarbia had no influence on ganglion blood flow. In addition, oxygen tension histograms were shifted to somewhat lower values than that of nerve. The unique microvascular characteristics of sensory ganglia and relative hypoxia may reflect the higher metabolic requirements of ganglion neurons, in comparison to the nerve trunk. It may be that these higher demands entrain both local rises in blood flow and induce autoregulation. Recently, these findings were

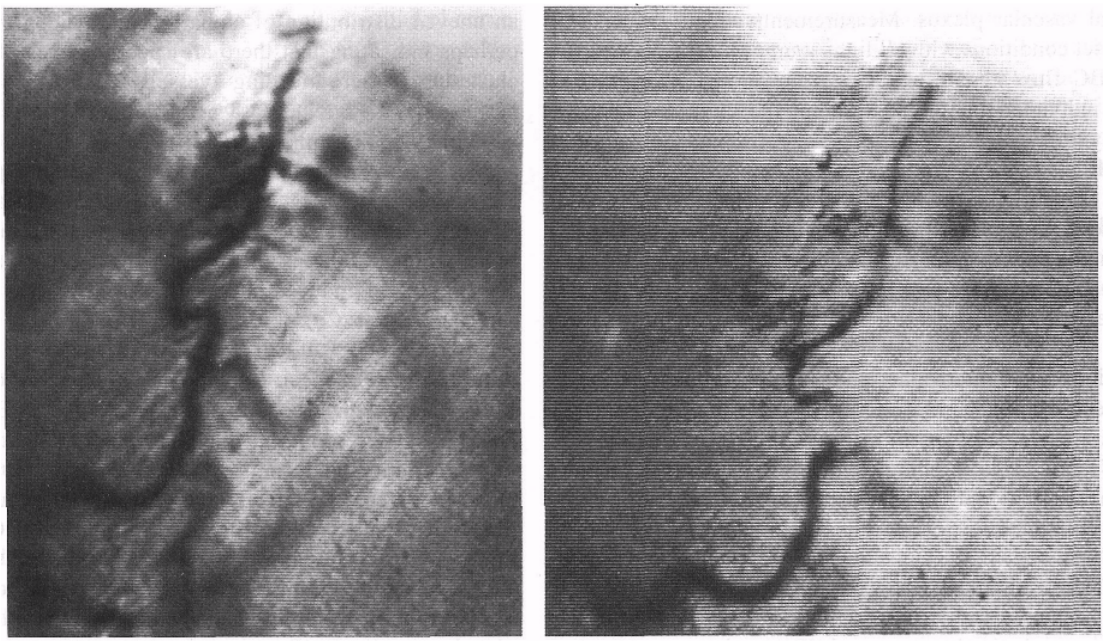

Figure 1. *Segmental vasoconstriction of an epineural microvessel exposed to bathing with norepinephrine. The photomicrograph on the left precedes treatment, and that on the right is after norepinephine. Uneven vasoconstriction suggested an uneven distribution of a-adrenergic receptors along the vessel. From Ref 9, with permission.*

verified using another technique for measuring blood flow, uptake of labelled iodoantipyrene (38).

ARE INJURED PERIPHERAL NERVES ISCHEMIC?

• An expected response of the peripheral nerve to traumatic injury, as in other tissues, is ischemia. Ischemia might arise from local disruption of the epineurial or endoneurial circulation with local thrombosis, endothelial swelling, granulocyte plugging or actual physical interruption of microvessels (2, 39). These events might result in no reflow and continuing fiber damage as occurs in prolonged nerve ischemia associated with vascular ligation (40). An additional problem following trauma might be the develop ment of endoneurial edema with microvascular compression. In the central nervous system, subarachnoid hemorrhage from aneurysmal rupture is also associated with intense cerebral vasospasm. In the spinal cord, acute compression injury is associated with cord ischemia (41). Some studies of acute mechanical injury of peripheral nerve by compression or crush postulated that ischemia was also an important mecha nism of injury (14, 42, 43). The issue is of obvious impor tance, because ischemia might have considerable impact on the local regenerative milieu.

We studied the impact of local nerve crush of the sciatic nerves of rats on EBF measured by hydrogen clearance polarography directly at the site of injury (44). The crush was administered by a hemostatic clamp applied across the nerve along a 3 mm segment for up to 3 hours with control rats having undergone nerve exposure only. Both precrush and postcrush measurements were made. Contrary to expectation, nerve crushes of duration 30 seconds, 2 hours or 3 hours were not associated with evidence of local ischemia either immediately after the clamp was removed or up to 2 hours later, when endothelial swelling and granulocyte plugging might have been expected to occlude vessels. There was a slight trend toward higher blood flow in the crushed nerve in comparison to controls after 2 hours. Similarly, oxygen tensions within the endoneurial space did not decline. The findings suggested that local ischemia was not an expected consequence of focal nerve injury, and that other factors must compensate for any direct microvessel damage. There might be the opening of previously closed vessels, release of local vasodilators or compensation for injury by the rich anasto-motic supply of the nerve trunk. Measurements of local EBF made at later time points after injury, 24 and 48 hours (45), indicated that flow increased beyond the normal range, a change that could be reversed by topically applying a specific

CGRP receptor antagonist, hCGRP (8-37). This was the first evidence we encountered that local vasodilators, in particular CGRP, may have a significant impact on the microcirculation of injured peripheral nerves. The findings also indicated that the peripheral nerve trunk compensated for injury related microvessel damage by peptidergic vasodilation or hyperemia.

NEUROGENIC INFLAMMATION OF THE PERIPHERAL NERVE TRUNK

• Work in other tissues has demonstrated that peptidergic unmyelinated nerve fibers participte in inflam matory processes (46-48). Perivascular peptidergic fibers transmit nociceptive information centrally, but also deliver peptides locally to the injury site. These local peptides in turn augment the inflammatory process as part of the process of neurogenic inflammation. SP, the first peptide studied, is a local vasodilator, induces plasma extravasation to cause edema, and degranulates mast cells. CGRP, a more recently recognized peptide, is a more potent vasodilator than SP and is coreleased with it. Histamine from mast cells, and perhaps other substances, may in turn further depolarize unmyelinated axons activating further peptidergic fibers. Neurogenic inflammation likely involves the axon reflex whereby fiber depolarization spreads in a retrograde fashion to other branches of the same parent axon to widen the territory involved (46, 49). Thus, peptidergic afferents contribute to the Gallenus features of inflammation: *tumor* (swelling, or edema from plasma extravasation), *rubor* and *color* (redness and heat from vasodilation), and *dolor* (pain from activation of nociceptive unmyelinated fibers).

Immunohistochemical and pharmacological work has demonstrated that vasa nervorum, like other tissues, are also innervated by peptidergic fibers capable of potentiating an inflammatory process (10, 50, 51). That the peripheral nerve trunk is self innervated and capable of participating in its own inflammatory processes is a novel concept, and is interesting. We tested neurogenic inflammatory vasodilation in sciatic nerves of rats by acutely depolarizing peptidergic perivascular nerve terminals innervating vasa nervorum with capsaicin. Capsaicin treatment in long term experiments results in a retrograde destruction of SP- and CGRP-containing unmyelinated fibers, but in the short term releases these peptides locally into the perivascular space (48). Capsaicin evoked a brisk, potent and sustained vasodilatory response in the sciatic nerve (Fig. 2, 3). Moreover, this response could be blocked by inhibitors of the CGRP receptor, the SP receptor, by prior capsaicin desensitization, by morphine, antihistamines, and cromolyn sodium, an inhibitor of mast cell degranulation (10,50) (Fig. 3). Since the full response was so sensitive to a variety of approaches, we postulated that its full expression involved the participation of CGRP particularly, but also SP, mast cells, and histamine. The sensitivity to morphine suggested that local opioid receptors, perhaps on peptidergic fibers might inhibit peptide releases. Opioids also inhibit activation of smooth muscle adenyl cyclase (52-54), an important direct action that may dampen neurogenic inflammation. Prior section of the nerve proximally had no influence on capsaicin hyperemia and indicated that its action was truly local. In contrast, section of the nerve 2 weeks before the experiment eliminated the capsaicin hyperemic response (Zochodne and Ho, unpublished data). By two weeks, section of the proximal nerve trunk resulted in axonal degeneration of the nerve terminals innervating the vasa nervorum, eliminating the capsaicin response. In contrast, section of the nerve trunk at the time of the experiment had no immediate influence on the capsaicin response because the terminals had not degenerated yet. The findings suggest that vasa nervorum are indeed innervated by the same parent nerve trunk they supply. There is similar evidence that adrenergic fibers innervating vasa nervorum arise from the parent nerve trunk (12, 55). Stimulation of the nerve trunk or its dorsal root supply appears to preferentially activate peptidergic perivascular fibers, resulting in vasodilation of vasa nervorum that depends on the stimulation frequency and is blocked by a CGRP receptor antagonist (11, 56).

RESPONSE TO INJURY: EARLY STAGES IN THE PROXIMAL STUMP

• Following peripheral nerve crush or transection, there are interesting and distinct changes that occur in both the distal denervated stump and the proximal stump. In the distal stump, local events prepare the environment for the entry of newly sprouted axons, including the formation of Schwann-basement membrane cell tubes or bands of Bungner, elaboration of growth factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) by Schwann cells, and changes in the characteristics of the basal lamina. These events occur in the context of breakdown of myelin and axons from the previously sectioned nerves, disruption of the blood-nerve barrier and the invasion of macrophages. These events are of obvious importance, but have been reviewed in detail by others and will not be discussed further here (57, 58). .

Relatively less attention has been paid to local events in the proximal stump of injured nerves and their relationship to regeneration. The classical monograph by Cajal (59) on degeneration and regeneration in the nervous system and detailed ultrastructural studies by Morris *et al* (60-63) both outlined important features of proximal nerve stumps where early regenerative events begin. Axonal endbulbs described by these authors resulted from the enlargement of transected

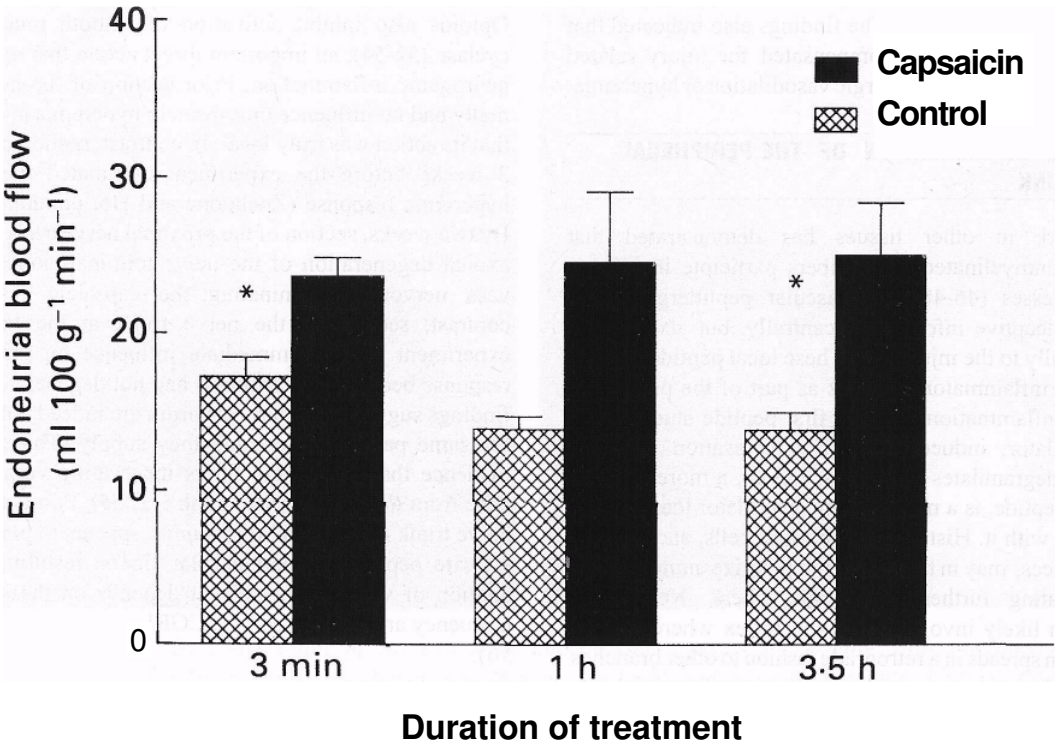

Figure 2. *Sciatic nerve endoneurial blood flow (EBF) in rats treated with epineurial capsaicinfor varying times. EBF was significantly increased by capsaicin, accounted for by local release of SP and CGRP from perivascular fibers innervating epineural vessels. From Ref 10, with permission.*

axons with accumulation of axoplasmic material. While axonal endbulbs have generally been considered as interesting pathological phenomena, we have suggested that they represent an important local repository of active molecules including enzymes and peptides. One such example is CGRP that is deposited in proximal nerve stumps of transected nerves within 48 hours of transection (23). Dramatic evidence of CGRP accumulation in distended axonal endbulbs was demonstrated by immunohistochemical labelling of proximal stumps of transected sciatic nerves in rats. In longitudinal sections of the stumps, CGRP immunoreactivity colocalized with neurofilament accumulation in the distal ends of individual axons (64). CGRP accumulation peaked by 48 hours then declined by 7 days. While CGRP acts as a neurotransmitter, it is also a very potent vasodilator. Its local accumulation in proximal nerve stumps paralleled rises in local blood flow in turn reversed by a specific CGRP receptor antagonist, hCGRP (8-37). Later, when CGRP accumulation in endbulbs was no longer demonstrable, hyperemia was no longer reversed by the blocking peptide. These findings suggested several interesting possibilities. Firstly, they demonstrated possible physiological relevance of axonal endbulbs. At early time points after axonal transection, these endbulbs must allow some egress of CGRP to dilate local vessels. In further work, it was demonstrated that peptide accumulation, as demonstrated with CGRP, also occurred with SP, NPY and galanin (Zochodne and Cheng, unpublished data). Interruption of axoplasmic transport, by a second, more proximal axonal transection, completely eliminated peptide accumulation (64, 65). We have termed this pattern of early peptide accumulation at the ends of transected axons and in the extracellular space peptide dumping. Unlike other tissues, the nerve trunk may actually be able to participate in its own repair in a very interesting way: disrupted local axons may add neuropeptides, beyond those released by their own perivascular afferents, into the injury milieu, and continue to deposit them by local axoplasmic transport. Some anterogradely transported peptides may be simply deposited and stored in swollen

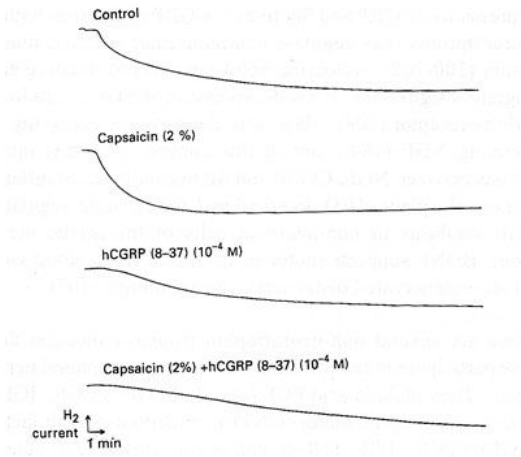

Figure 3. *Examples of hydrogen microelectrode clearance curves indicating changes in slope with various interventions applied topically over the epineurial vascular plexus. Actual blood flow values are calculated by curve fitting as described in the text. Note the faster washout (and flow) associated with acute SP and CGRP release from perivascular fibers exposed to capsaicin. This is blocked by a CGRP receptor antagonist hCGRP (8-37) on the bottom curve. The second from the bottom curve is the influence of the CGRP antagonist alone, compared to the control (top) curve. The curve flattening indicates vasoconstriction, implying that CGRP normally tonically dilates vasa nervorum. From Ref 10, with permission.*

axonal endbulbs without reaching the extracellular space (60). Similarly, the function of extracellular dumped peptides is unclear, besides the obvious microvascular vasodilator actions of SP and CGRP, SP being also a chemoattractant (47), CGRP may be a mitogen for Schwann cells and other cellular elements, acting through adenyl cyclase and cAMP (66, 67). Both peptides degranulate mast cells (47). SP and histamine from mast cells both cause plasma extravasation, a potentially important route for ingress of trophins from plasma to the regenerative milieu. Galanin, in contrast, has been considered to have analgesic actions (68). While dumping of peptides appears to occur at the injury milieu, parallel alterations in peptide expression occur in perikarya of motor and sensory axons. CGRP expression, for example, rises in motor neurons but declines in sensory perikarya (69-74).

It is conceivable that dumping of peptide neurotransmitters and perhaps selected enzymes may be an early phenomenon of other types of axonal interruption such as that following central nervous system white matter injury. Two isoforms of the NOS, the neuronal (nNOS), and eNOS, also appear to be transported and deposited in proximal nerve stumps (35, 64). Like CGRP, they can be colocalized with neurofilament in axonal endbulbs of the tips of the proximal stumps, and their delivery to these stumps is interrupted by a second more proximal transection. Moreover, broad spectrum pharmacological inhibition of NOS, like the CGRP antagonist, reversed hyperemia in inj ured proximal stumps. This evidence suggests that NO is locally generated by accumulated NOS in axonal endbulbs, and is released into the injury milieu where it dilates local microvessels. The potential roles of NO in the injury milieu will be considered further below.

The next major phase of peripheral nerve repair is recruitment of blood-borne inflammatory cells, mainly macrophages (75). Macrophages deliver a variety of important trophins to the regenerative milieu, including basic fibroblast growth factor (bFGF), granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor-a (TGF-a), insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), interleukin-8, and others (76). Macrophages release bFGF from bound heparin through release of proteases (76), and stimulate the production of NGF in denervated Schwann cells through the action of interleukin-1 (47, 77). They may also synthesize NO through inducible NOS (iNOS). In neuromas, there are prominent and persistent infiltrations of macrophages that could have direct actions on denuded axonal membranes (78). Suppression of macrophage function delays regeneration (79).

Based on immunohistochemical criteria, we have considered local proliferation the third phase of the proximal stump response, following peptide dumping and macrophage invasion. Angiogenesis and further vasodilation from histamine liberated by mast cells might sustain hyperemia in injured nerve proximal stumps after CGRP levels decline. Coordinated proliferative events in experimental nerve injuries appear after 3-4 days in injured nerve and involve endothelial cells, mast cells, axonal sprouts, Schwann cells, fibroblasts, and perhaps perineurial cells (22, 60-63, 80-83). Outgrowth from the proximal stump allows reconstitution of the nerve trunk (84,85). Some of these events are illustrated on Fig. 4.

Mast cells are likely to be important elements in the repair and inflammatory response of peripheral nerves after injury. Rises in mast cell numbers from local proliferation (rather than recruitment) have been demonstrated in several animal models of peripheral nerve damage and as well in human neuropathies and neuromas (83, 86-90). Local degranulation

and release of histamine may be important in opening the blood-nerve barrier after injury (87). This in turn may allow endoneurial constituents to be influenced by blood-borne trophins and other agents during degeneration, and regeneration (91). Mast cells also contain a variety of other important constituents including growth factors (see below), proteolytic enzymes, tumor necrosis factor-a (TNF-a), interleukins, and other cytokines (92). Close apposition between some mast cells and macrophages and other cell types may represent a unique signalling pathway, important in the injury environment (reviewed in 92).

The neurotrophins (NGF, BDNF, NT-3, NT-4/5 and NT-6) have been examined by several laboratories. Although NGF for example, may not directly influence regenerative fiber sprouting, it does promote collateral sprouting, and influences the architecture of the regenerating nerve trunk, including its neovasculature (93-96). There may be important but subtle changes in the peptide characteristics of sprouting axons (a different matter than peptide dumping described above) that are influenced by neurotrophins and other growth factors. For example rat sciatic neuroma axon sprouts express CGRP, SP, and NPY (97-99), and a one year old mechanosensitive human neuroma studied in our lab (90) had enhanced expression of CGRP and SP. In turn, CGRP expression within motor sprouts may regulate neuromuscular junction innervation (100-102). Autocrine Schwann cell proliferative and migratory signals involve self-expression of NGF and its low-affinity receptor (103). Mast cells also have the capability of secreting NGF (104), and in this context important interactions between NGF, CGRP and SP in conditions of inflammation take place (105). Interleukin-1 for example, regulates NGF synthesis in non-neuronal cells of the sciatic nerve (106). BDNF supports motor neurons and rises, along with NT-4, in denervated distal sciatic nerve stumps (107).

There are several non-neurotrophin trophic molecules that may participate in the proliferative phase of peripheral nerve repair. They include acid FGF (aFGF), bFGF, VEGF, IGFs, ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), PDGF, TGF, TNF-a, and presumably several others. Many of these factors have been demonstrated to promote cutaneous healing and angiogenesis (108-112), and it is very likely that they would also support peripheral nerves (113).

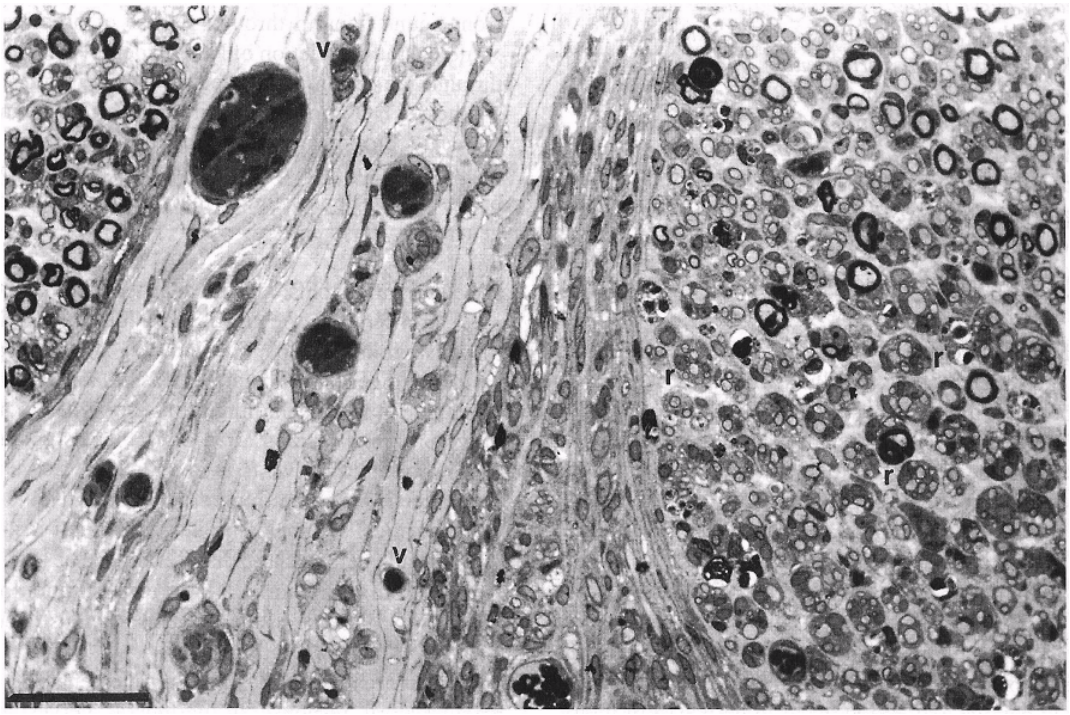

Figure 4. Photomicrograph from the proximal stump of a rat sciatic nerve transected 14 days earlier. Note the large numbers of regenerative units containing myelinated axonal sprouts (r). These sprouts splay apart layers of the previous perineurium. Multiple small vessels, several likely newly formed (v) are also observed. Bar, 40 jj,m.

Among them, direct evidence of a benefit on nerve regeneration has been demonstrated with bFGF (114), aFGF (115, 116), IGF-II and insulin (117), and CNTF (118,119). In distal denervated nerve stumps, Schwann cells elaborate IGF-I and IGF-II (120), TGF-PI (121), and EOF receptors (122). Some of these factors may act as autocrine Schwann cell mitogens, chemoattractants for macrophages, or modulators of cell adhesion molecules (121). EGF receptor rises to particularly high levels at the injury site itself, whereas TGF-J31 appears to be transported distally from axotomized sensory neurons (112). Ischemia may also influence their activity since peripheral nerves with ischemic axonal degeneration regenerate more slowly (123, 124). Some of the non-neurotrophin growth factors may benefit nerve regeneration by supporting angiogenesis. This possibility has received little attention. Among the known potent angiogenic factors, those that might act on peripheral nerve in this fashion are bFGF (108), TGF-PI (110), VEGF (125), and PDGF (108).

The next challenge to the peripheral nerve repair process is regrowth through the distal nerve stump. Peripheral nerve surgeons have realized that reconnection of severed nerve trunks is best carried out earlier rather than later, because axonal regrowth through long term denervated nerve trunks becomes limited. Part of this change in the microenvironment for regeneration may be the loss of Schwann cell basement membrane columns (bands of Bungner), loss or atrophy of Schwann cells themselves, loss of continuous basement membrane scaffolding, collagen remodeling and deposition, or loss of integrin-pl (126-129). Another possibility is that long term denervated trunks become ischemic and unable to support the metabolic demands of nerve sprouts (130).

In summary, four main themes (Fig. 5) appear to be woven into the peripheral nerve repair process: *(i)* reactive hyperemia and peptide dumping, *(ii)* a chemotactic and recruitment phase; *(Hi)* a proliferative phase, and *(iv)* a reinnervation phase requiring regrowth through the distal nerve stump. Later events in the injury milieu likely depend on whether there is successful regeneration to a distal nerve trunk, or target, resulting in reconstitution of the previous appropriate structure of the nerve. If this does not occur, local expansion at and beyond the site of transection results in the formation of a neuroma. What finally arrests neuroma growth is unknown, but it could occur because of ischemia from local fibrosis and vessel entrapment.

THE ROLE OF NITRIC OXIDE

• One of the important players in the inflammatory cascade of events that develops within the injury milieu may

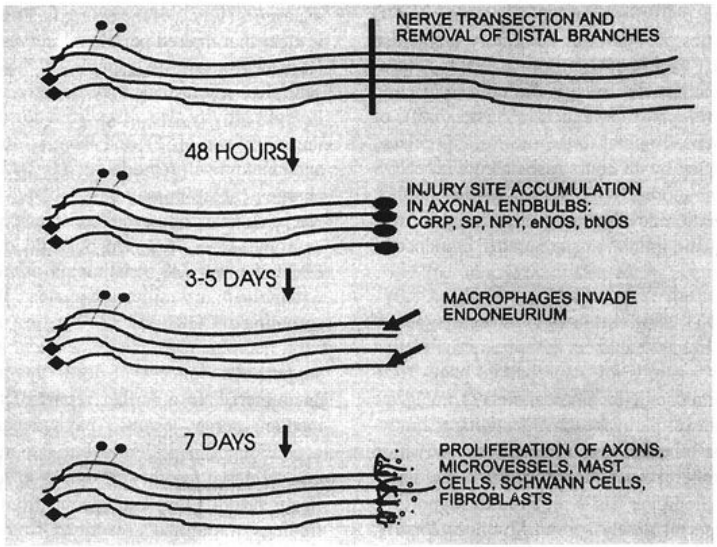

***Figure** 5. As suggested simplified version of early events in the regenerative milieu of transected peripheral nerves.*

be NO and the respective NOS mentioned above (131-133). NO might have several potential actions within peripheral nerves. It may act as the final pathway for vasodilation by CGRP, SP and other locally expressed peptides. nNOS could be deposited by sectioned axons to within the injury zone, because increased expression in dorsal root ganglion cells and anterior horn cells following axotomy has been recognized (134, 135). Finally, macrophage recruitment might bring with it high levels of iNOS (136) whereas inflammatory conditions might result in the expression of iNOS in Schwann cells (137, 138).

We examined the proximal nerve stumps of transected rat sciatic nerves for evidence of NO activity and synthesis (35, 64). Several interesting and unexpected observations were made. Local changes in blood flow provided an index of NO presence, otherwise very difficult to detect in small nerve trunks. Injury-related hyperemia, or rises in nerve blood flow, noted to be CGRP-dependent as described above, were also highly sensitive to the broad spectrum NOS inhibitors L-NAME and L-NNA. The NOS inhibitors reversed hyperemia at early time points, as observed with the CGRP antagonist, but also at later time points when CGRP played no apparent role (see above). The findings provided a signature of NO presence. Immunohistochemical studies demonstrated that there was prominent staining of eNOS, and to a lesser extent of nNOS within axonal endbulbs. At later time points, some macrophages appeared to express iNOS, although expression was infrequent. An assay of NOS activity, using labelled arginine to citrulline conversion, identified rises in NO generation at later times. Levy in our laboratory (139) also observed a very similar pattern of change proximal to sutures placed around the sciatic nerve in the chronic constriction model, originally developed by Bennett and Xie (140), of sciatic neuropathic pain. Proximal to the sutures, NO-related hyperemia was identified by 48 hours associated with eNOS and some nNOS immunoreactivity. Distal to the sutures, where axonal degeneration develops in this model, there was both pharmacological and immunohistochemical evidence of increased iNOS activity.

What actions might NO exert within both of these types of nerve injury? NO might enhance ectopic activity that contributes to the sensations of neuropathic pain. NOS inhibitors have systemic and local (near nerve) analgesic actions (141), and painful paravascular injections of brady-kinin or hyperosmolar solutions in humans require activation of NOS (142). Another possibility is that NO-mediated vasodilation of local blood vessels is required to support the metabolic requirements of newly sprouting axons. Finally, NO might be detrimental to the regenerative milieu because of its conversion to the peroxynitrite, a product with known cytoxic properties that induces growth cone collapse (143, 144). We administered L-NAME to mice for 10 days after having undergone transection of the sciatic nerve (145). Controls received the inactive enantiomer, D-NAME. Regeneration was assessed by the recovery of the compound muscle action potential, or M potential of sciatic-tibial innervated distal foot muscles and by the numbers and caliber of myelinated fibers at a fixed distance beyond the transection site by 10 weeks. Our working hypothesis was that NOS ; inhibition might impede regeneration by reversing injury- ' related rises in local nerve blood flow. Contrary to expectation, however, initial investigation suggested that NOS inhibition was associated with earlier and more complete recovery of the M potential and evidence of more mature myelinated fiber regeneration by 10 weeks. While this work requires confirmation, it may be that excessive local liberation of NO, perhaps in zones where NOS is concentrated, impairs regeneration by damaging sprouts or regenerating fibers. Further study of the possible actions of NO and NOS in the injury milieu, and their interaction with cytokines and other inflammatory actors will be of considerable interest.

NEUROPATHIC PAIN: POSSIBLE MECHANISMS

- While the impact of local events in the injury milieu of proximal nerve stumps on regenerative success is still unclear, local events might also generate neuropathic pain. Pain is a common problem among patients with traumatic nerve injury or neuropathies such as that due to diabetes. It likely develops because of plastic changes at several anatomical levels of the neuraxis. From the previous works, it is clear that injured peripheral nerves, particularly those that form neuromas, are capable of directing ectopic impulses centrally, where they may be perceived as pain (146-150). Ectopic activity also arises from dorsal root ganglia central to the injury site (151), and there is dorsal horn physiological and anatomical remodeling (152-154). Local ectopic dis charge generation may be facilitated by the accumulation of Na^+ channels, observed both in experimental and human neuromas (155, 156). The possible local roles of NO, CGRP, SP, and mast cell constituents on ectopic discharge firing within the injury milieu are unclear. We reported evidence of sprouting of CGRP and SP fibers in a mechanosenstive human sural neuroma (90). No evidence of adrenergic sprouting, to support the concept of local sympathetic activation was encountered. In a further report of two patients with local median nerve lesions, pain accompanied evidence of activation of peptidergic but not adrenergic fibers in the injured nerve territory, resulting in highly localized redness (from vasodilation) and edema (from plasma extravasation), findings traditionally ascribed to reflex sympathetic dys trophy (157). The role of sympathetic activation of neuro pathic pain, however, cannot be discarded since adrenergic agonists enhance neuroma discharges. Following nerve

injury, sympathetic sprouting occurs in dorsal root ganglia, a possible site of adrenergic modulation (158).

In diseased or injured but otherwise intact nerve trunks, neurogenic inflammation might contribute toward pain gene ration. This mechanism might be important, for example, in inflammatory demyelinating polyneuropathies. In more seve re disruptions of the nerve trunk, it is possible that local peptide dumping at early time points, or sprouting of CGRP and SP fibers later, help to generate ectopic discharges. The possible role of NO has been discussed above. We have postulated that there may be local actions of opioids in the injury milieu that could influence ectopic discharges. Whereas nerve injury-related hyperemia could be reversed. by a CGRP antagonist and NOS inhibitors, it could also be reversed by several opioid agonists (159). Opioids are also potent inhibitors of neurogenic inflammation, but it is uncertain whether they inhibit afferent ectopic discharges. Their influence on micro vessels suggests that opioid receptors may be expressed locally at sites of peripheral nerve injury. The interacting local roles of peptides, NO, growth factors, opioids, cytokines, and chemokines in the milieu of injured peripheral nerves remain to be defined. They offer both the clinician and the basic scientist avenues to explore, because understanding these mechanisms might bear on the two fundamental complications of peripheral nerve injury: functional disability from poor regeneration, and pain.

CONCLUSION

• There are many facets of the microenvironment of injured and regenerating peripheral nerves that we do not completely understand yet. We do not know, for example, how important local microvascular responses and subsequent angiogenesis are to the success of fiber regrowth. The possi bilities, however, of a major role for peptides, such as SP and CGRP, NO, and local opioid receptors offer several exciting avenues to explore. It may be that their actions on nerve microvessels from injured nerves simply reflect a much broader scope of action in the repair process of nerve.

ACKNOWLEDGEMENTS

• Brenda Bake provided expert secretarial assistance. Douglas Zochodne is a Medical Scholar of the Alberta Heritage Foundation for Medical Research and has received operating grant support from the Muscular Dystrophy Association of Canada and the Medical Research Council of Canada. 12.

1. Lyons WR, Woodhall B, Saunders WB, editors. *Atlas of Peripheral Nerve Injuries.* Philadelphia, 1949.

2. Woodhall B, Davis C. Changes in the arteriae nervorum in peripheral nerve injuries in man. *J Neuropathol Exp Neural* 1950; 9:335-343.

3. Bell MA, Weddell AG. A descriptive study of the blood vessels of the sciatic nerve in the rat, man and other mammals. *Brain* 1984; 107:871-898.

4.Appenzeller O, Dhital KK, Cowen T, Burnstock G. The nerves to blood vessels supplying blood to nerves: the innervation of vasa nervorum. *Brain Res* 1984; 304:383-386.

5.Dhital K, Appenzeller O. Innervation of vasa nervorum. In: Burnstock G, Griffith SG, editors. In: *Nonadrenergic innervation of blood vessels.* CRC Press, Boca Raton, 1988; 191-211.

6. Kihara M, Low PA. Regulation of rat nerve blood flow: role of epineurial alpha-receptors. *JPhysiol* 1990; 422: 145-152.

7. Rechthand E, Hervonen A, Sato S, Rapoport SI. Distribution of adrenergic innervation of blood vessels in peripheral nerve. *Brain Res* 1986; 374:185-189.

8. Zochodne DW, Huang ZX, Ward KK, Low PA. Guanethidine-induced adrenergic sympathectomy augments endoneurial perfusion and lowers endoneurial microvascular resistance. *Brain Res* 1990; 519:112-117.

9. Zochodne DW, Low PA. Adrenergic control of nerve blood flow. *Exp Neural* 1990; 109: 300-307.

10. Zochodne DW, Ho LT. Influence of perivascular peptides on endoneurial blood flow and microvascular resistance in the sciatic nerve of the rat. *J Physiol* 1991; 444: 615-30.

11. Zochodne DW, Ho LT. Stimulation-induced peripheral nerve hyperemia: mediation by fibers innervating vasa nervorum? *Brain Res* 1991; 546: 113-118.

Kummer W, Seifert E, Schadel A. Innervation of epi- and endoneurial compartments of rat facial, vagus and sciatic nerves as studied by double-labeling immunofluorescence. *Acta Anat* 1994; 149: 264-271.

13. Lundborg G, Branemark P-I. Microvascular structure and function of peripheral nerves. *Adv Microcirc* 1968;

14. Lundborg G. Structure and function of the intraneural microvessels as related to trauma, edema formation, and nerve function. *J Bone Joint SurgAm* 1975; 57: 938-948.

15. Zochodne DW. Is early diabetic neuropathy a disorder of the dorsal root ganglion? A hypothesis and critique of some current ideas on the etiology of diabetic neuropathy. *J Per Nerv Syst* 1996; 1: 1-12. . . .,k,

16. Low PA, Lagerlund TD, McManis PG. Nerve blood flow and oxygen delivery in normal, diabetic, and ischemic neuropathy. *Int Rev Neurobiol* 1989; 31: 355-438.

17. Day TJ, Lagerlund TD, Low PA. Analysis of H_2 clearance curves used to measure blood flow in rat sciatic nerve. *J Physiol* 1989; 414: 35-54.

18. McManis PG, Low PA, Yao JK. Relationship between nerve blood flow and intercapillary distance in peripheral nerve edema. *Am J Physiol* 1986; 251: E92-E97.

19. Stosseck K, Lubbers DW. Local generation and measurement of hydrogen in tissue by means of bare platinum electrodes. In: Kessler M, Bruley DF, Lark LC, Lubbers DW, Silver IA, Strauss J, editors. *Oxygen supply.* University Park, Baltimore, 1971; 113-114.

20. Kalichman MW, Lalonde AW. Experimental nerve ischemia and injury produced by cocaine and procaine. - *Brain Res* 1991; 565:34-41.

21. Rundquist I, Smith QR, Michel ME, Ask P, Oberg PA, Rapoport SI. Sciatic nerve blood flow measured by laser Doppler flowmetry and [14C]iodoantipyrme. *Am J Physiol* 1985; 248: H311-H317

22. Zochodne DW, Nguyen CA. Angiogenesis in experimen tal neuromas. J^na?1997;190:23-30. . . . . .

23. Zochodne DW, Allison JA, Ho W, Ho LT, Hargreaves K, Sharkey KA. Evidence for CGRP accumulation and activity in experimental neuromas. *Am J Physiol* 1995;
. 268:H584-H590.

24. Low PA, Tuck RR. Effects of changes of blood pressure, respiratory acidosis and hypoxia on blood flow in the sciatic nerve of the rat. *J Physiol* 1984; 347: 513-524.

25. Takeuchi M, Low PA. Dynamic peripheral nerve metabolic and vascular responses to exsanguination. *Am J Physiol* 1987; 253:E349-E353.

26. Rechthand E, Sato S, Oberg PA, Rapoport SI. Sciatic nerve blood flow response to carbon dioxide. *Brain Res* 1988; 446:61-66.

27. Zochodne DW, Ho LT, Gross PM. Acute endoneurial ischemia induced by epineurial endothelin in the rat sciatic nerve. *Am J Physiol* 1992; 263: H1806-H1810.

28. Zochodne DW, Cheng C, Sun H. Diabetes increases sciatic nerve susceptibility to endothelin-induced ischemia. *Diabetes* 1996; 45: 627-632.

29. Zochodne DW, Ho LT. Vasa nervorum constriction from substance P and calcitonin gene-related peptide antagonists: sensitivity to phentolamine and nimodipine. *Regul Pept* 1993; 47: 285-290.

30. Marshall I. Mechanism of vascular relaxation by the calcitonin gene-related peptide. *Ann NYAcad Sci* 1992; 657: 204-215.

31. Fiscus RR, Zhou HL, Wang X, Han C, Ali S, Joyce CD *et al.* Calcitonin gene-related peptide (CGRP)-induced cyclic AMP, cyclic GMP and vasorelaxant responses in rat thoracic aorta are antagonized by blockers of endo-thelium-derived relaxant factor (EDRF). *Neuropeptides* 1991; 20: 133-143.

32. Quayle JM, Bonev AD, Bray den JE, Nelson MT. Calcitonin gene-related peptide activated ATP-sensitive K^+ currents in rabbit arterial smooth muscle *via* protein kinase A. *J Physiol* 1994; 475:9-13.

33. Schini-Kerth VB, Fisslthaler B, Busse R. CGRP enhances induction of NO synthase in vascular smooth muscle cells *via* a cAMP-dependent mechanism. *Am J Physiol* 1994; 267: H2483-H2490.

34. Nelson MT, Huang Y, Brayden JE, Hescheler J, Standen NB. Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. *Nature* , 1990; 344: 770-773.

35. Zochodne DW, Levy D, Sun H, Cheng C, Lauritzen M, Rubin I. Nitric oxide and nitric oxide synthetase in injured peripheral nerve trunks [abstract]. *Can J Neural Sci* 1997; 24 (Suppl 1): S57.

36. Kihara M, Low PA. Vasoreactivity to prostaglandins of rat peripheral nerve. *J Physiol* 1995; 484: 463-467.

3 7. Zochodne D W, Ho LT. Unique micro vascular characteristics of the dorsal root ganglion in the rat. *Brain Res* 1991; 559: 89-93.

38. McManis PG, Schmelzer JD, Zollman PJ, Low PA. Blood flow and autoregulation in somatic and autonomic ganglia. Comparison with sciatic nerve. *Brain* 1997; 120: 445-449.

39. Schmid-Schonbein GW. Capillary plugging by granulocytes and the no-reflow phenomenon in the microcirculation. *Fed Proc* 1987; 46: 2397-2401.

40. Schmelzer JD, Zochodne DW, Low PA. Ischemic and reperfusion injury of rat peripheral nerve. *Proc Natl Acad Sci USA* 1989; 86: 1639-1642.

41. Fehlings MG, Tator CH, Linden RD. The relationships among the severity of spinal cord injury, motor and somatosensory evoked potentials and spinal cord blood flow. *Electroencephalogr Clin Neurophysiol* 1989; 74: 241-259.

42. Rydevik B, Lundborg G, Bagge U. Effects of graded compression on intraneural blood blow. An in vivo study on rabbit tibial nerve. *Am J Hand Surg* 1981; 6: 3-12.

43. Lundborg G, Myers R, Powell H. Nerve compression injury and increased endoneurial fluid pressure: a "miniature compartment syndrome". *JNeurolNeurosurg Psychiat* 1983; 46: 1119-1124.

44. Zochodne DW, Ho LT. Endoneurial microenvironment and acute nerve crush injury in the rat sciatic nerve. *Brain Res* 1990; 535: 43-48.

45. Zochodne DW, Ho LT. Hyperemia of injured peripheral nerve: sensitivity to CGRP antagonism. *Brain Res* 1992; 598: 59-66.

46. Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 1988; 24: 739-768.

47. Levine JD, Fields HL, Basbaum AI. Peptides and the primary afferent nociceptor. *J Neurosci* 1993; 13: 2273-2286

48. Maggi CA. Tachykinins and calcitonin gene-related peptide (CGRP) as cotransmitters released from peripheral endings of sensory nerves. *Prog Neurobiol* 1995; 45: 1-98.

49. Lembeck F. Sir Thomas Lewis's nocisensor system, histamine and substance P containing primary afferent nerves. *Trends Neurosci* 1983; 6106-6108.

50. Zochodne DW, Ho LT. Evidence that capsaicin hyperaemia of rat sciatic vasa nervorum is local, opiate-sensitive and involves mast cells. *JPhysiol* 1993; 468: 325-333.

51. Zochodne DW. Epineurial peptides: a role in neuropathic pain ? *Can J Neural Sci* 1993; 20: 69-72.

52. Cooper DM, Londos C, Gill DL, Rodbell M. Opiate receptor-mediated inhibition of adenylate cyclase in rat striatal plasma membranes. *J Neurochem* 1982; 38: 1164-1167.

53. Lambert DG, Atcheson R, Hirst RA, Rowbotham DJ. Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [3H]noradrenaline release from SH-SY5Y cells. *Biochem Pharmacol* 1993; 46: 1145-1150.

54. Sharma SK, Nirenberg M, Klee WA. Morphine receptors as regulators of adenylate cyclase activity. *Proc Natl Acad Sci USA* 1975; 72:590-594.

55. Koistinaho J, Wadhwani KC, Balbo A, Rapoport SI. Regeneration of perivascular adrenergic innervation in rat tibial nerve after nerve crush. *Acta Neuropathol* 1991; 81: 486-490.

56. Sato A, Sato Y, Uchida S. Blood flow in the sciatic nerve is regulated by vasoconstrictive and vasodilative nerve fibers originating from the ventral and dorsal roots of the spinal nerves. *Neurosci Res* 1994; 21:125-133.

57. Griffin JW, George EB, Hsieh S-T, Glass JD. Axonal degeneration and disorders of the axonal cytoskeleton. In: Waxman SG, Kocsis JD, Stys PK, editors.*The axon. Structure, function and pathophysiology.* Oxford University Press, Oxford 1995; 20: 375-390.

58. Bisby MA. Regeneration of peripheral nervous system axons. In: Waxman SG, Kocsis JD, Stys PK, editors. *The axon. Structure, function and pathophysiology.* Oxford University Press, Oxford, 1995; 28: 553-578.

59. Cajal SR. *Degeneration and regeneration in the nervous system.* Oxford University Press, Oxford, 1928.

60. Morris JH, Hudson AR, Weddell G. A study of

degeneration and regeneration in the divided rat sciatic nerve based on electron microscopy. III. Changes in the axons of the proximal stump. *ZZellforsch MikroskAnat* 1972; 124: 131-164.

61. Morris JH, Hudson AR, Weddell G. A study of degeneration and regeneration in the divided rat sciatic nerve based on electron microscopy. IV. Changes in fascicularmicrotopography, perineurium and endoneurial fibroblasts. *Z Zellforsch Mikrosk Anat* 1972; 124: 165-203.

62. Morris JH, Hudson AR, Weddell G. A study of degeneration and regeneration in the divided rat sciatic nerve based on electron microscopy. I. The traumatic degeneration of myelin in the proximal stump of the divided nerve. *Z Zellforsch Mikrosk Anat* 1972; 124: 76-102.

63. Morris JH, Hudson AR, Weddell G. A study of degeneration and regeneration in the divided rat sciatic nerve based on electron microscopy. II. The development of the "regenerating unit". *Z Zellforsch Mikrosk Anat* 1972; 124: 103-130.

64. Zochodne DW, Cheng C. Accumulation of NOS isoforms in early axonal end bulbs of transected peripheral nerves. *Soc Neurosci Abst* 1997; 23: 1415.

6 5. Kashihara Y, Sakaguchi M, Kuno M. Axonal transport and distribution of endogenous calcitonin gene-related peptide in rat peripheral nerve. *J Neurosci* 1989; 9: 3796-3802.

66. Trantor IR, Messer HH, Birner R. The effects of neuropeptides (calcitonin gene-related peptide and substance P) on cultured human pulp cells. / *Dent Res* 1995; 74: 1066-1071. .. . . . .

67. Cheng L, Khan M, Mudge AW. Calcitonin gene-related peptide promotes Schwann cell proliferation. *J Cell Biol* 1995; 129: 789-796.

68. Verge VM, Xu XJ, Langel U, Hokfelt T, Wiesenfeld-Hallin Z, Bartfai T. Evidence for endogenous inhibition of autotomy by galanin in the rat after sciatic nerve section: demonstrated by chronic intrathecal infusion of a high affinity galanin receptor antagonist. *Neurosci Lett* 1993; 149: 193-197.

6 9. Arvidsson U, Johnson H, Piehl F, Cullheim S, Hokfelt T, Risling M *et al.* Peripheral nerve section induces increased levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity in axotomized motoneurons. *Exp Brain Res* 1990; 79: 212-216.

70. Caldero J, Casanovas A, Sorribas A, Esquerda JE. Calcitonin gene-related peptide in rat spinal cord motoneurons: subcellular distribution and changes induced by axotomy. *Neuroscience* 1992; 48: 449-461.

71. Dumoulin FL, Raivich G, Streit W, Kreutzberg G. Differential regulation of calcitonin gene-related peptide (CGRP) in regenerating rat facial nucleus and dorsal root ganglion. *Eur J Neurosci* 1991; 3338-3342.

72. Hokfelt T, Zhang X, Wiesenfeld-Hallin Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci* 1994; 17: 22-30.

73. Streit WJ, Dumoulin FL, Raivich G, Kreutzberg GW. Calcitonin gene-related peptide increases in rat facial motoneurons after peripheral nerve transection. *Neur osci Lett* 1989; 101: 143-148.

74. Villar MJ, Wiesenfeld-Hallin Z, Xu XJ, Theodorsson E, Emson PC, Hokfelt T. Further studies on galanin-, substance P-, and CGRP-like immunoreactivities in primary sensory neurons and spinal cord: effects of dorsal rhizotomies and sciatic nerve lesions. *Exp Neural* 1991; 112: 29-39.

75. Vass K, Hickey WF, Schmidt RE, Lassmann H. Bone marrow-derived elements in the peripheral nervous system. An immunohistochemical and ultrastructural investigation in chimeric rats. *Lab Invest* 1993; 69:275-282.

76. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *J Leukoc Biol* 1994; 55:410-422.

77. Heumann R, LindholmD, Bandtlow C, MeyerM, Radeke MJ, Misko TP *et al.* Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. *Proc Natl Acad Sci USA* 1987; 84: 8735-8739.

78. Frisen J, Risling M, Fried K. Distribution and axonal relations of macrophages in a neuroma. *Neuroscience* 1993; 55: 1003-1013.

79. Tanaka K, Zhang QL, Webster HD. Myelinated fiber regeneration after sciatic nerve crush: morphometric

observations in young adult and aging mice and the effects of macrophage suppression and conditioning lesions. *Exp Neural* 1992; 118: 53-61.

80. Pellegrino RG, Politis MJ, Ritchie JM, Spencer PS. Events in degenerating cat peripheral nerve: induction of Schwann cell S phase and its relation to nerve fibre degeneration. *J Neurocytol* 1986; 15: 17-28.

81. Podhaj sky RJ, Myers RR. The vascular response to nerve transection: neovascularization in the silicone nerve regeneration chamber. *Brain Res* 1994; 662: 88-94.

82. Weis J, May R, Schroder JM. Fine structural and immunohistochemical identification of perineurial cells connecting proximal and distal stumps of transected peripheral nerves at early stages of regeneration in silicone tubes. *Acta Neuropathol* 1994; 88: 159-165.

83. Zochodne DW, Nguyen C, Sharkey KA. Accumulation and degranulation of mast cells in experimental neuromas. *Neurosci Lett* 1994; 182: 3-6.

84. Thomas PK. The cellular response to nerve injury. 1. The cellular outgrowth from the distal stump of transected nerve. *J Anat* 1966; 100: 287-303.

85. Thomas PK. The cellular response to nerve injury. 2. Regeneration of the perineurium after nerve section. *J Anat* 1967; 101: 45-55.

86. Olsson Y. Mast cells in the nervous system. *IntRev Cytol* 1968; 24: 27-70.

87. Olsson Y, Reese TS. Permeability of vasa nervorum and perineurium in mouse sciatic nerve studied by fluorescence and electron microscopy. *J Neuropathol Exp Neurol* 1971; 30: 105-119.

88. Dal Toso R, Fabris M, Romanello S, Petrelli L, Buriani A, Alexandre A *et al.* Nerve growth factor immunoreactive mast cells in human peripheral nerve. *Soc Neuro sci Abst* 1994; 20: 953.

89. Olsson Y. Vascular permeability in the peripheral nervous system. In: Dyck PJ, Thomas PK, Lambert EH, Bunge RB, editors. *Peripheral neuropathy.* W.B. Saun-ders, Toronto, 1984; 27.

90. Zochodne DW, Cheng C, Theriault M, Sutherland G. Peptides and neuromas: calcitonin gene-related peptide, substance P and mast cells in a mechanosensitive human sural neuroma. *Muscle Nerve* 1997; 20: 875-880.

91. Seitz RJ, Reiners K, Himmelmann F, Heininger K, Hartung HP. Toyka KV. The blood-nerve barrier in Wallerian degeneration: a sequential long-term study. *Muscle Nerve* 1989; 12: 627-635.

92. Dines KG, Powell HC. Mast cell interactions with the nervous system: relationship to mechanisms of disease. *J Neuropathol Exp Neurol* 1997; 56: 627-640.

93. Doubleday B, Robinson PP. The effect of NGF depletion on the neurotropic influence exerted by the distal stump following nerve transection. *J Anat* 1995; 186: 593-605.

94. Spector JG, Lee P, Derby A, Frierdich GE, Neises G, Roufa DG. Rabbit facial nerve regeneration in NGF-containing silastic tubes. *Laryngoscope* 1993; 103: 548-558.

95. Diamond J, Foerster A, Holmes M, Coughlin M. Sensory nerves in adult rats regenerate and restore sensory function to the skin independently of endogenous NGF. *J Neurosci* 1992;12: 1467-1476.

96. Diamond J, Holmes M, Coughlin M. Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. *J Neurosci* 1992; 12: 1454-1466.

97. Frisen J, Risling M, Theodorsson E, Fried K. NPY-like immunoreactivity in sensory nerve fibers in rat sciatic neuroma. *Brain Res* 1992; 577: 142-146.

98. Fried K, Brodin E, Theodorsson E. Substance P-, CGRP- and NPY-immunoreactive nerve fibers in rat sciatic nerve-end neuromas. *Regul Pept* 1989; 25: 11-24.

99. Fried K, Frisen J. End structure and neuropeptide immunoreactivity of axons in sciatic neuromas following nerve section in neonatal rats. *Exp Neurol* 1990; 109: 286-293.

100. Lu B, Fu WM, Greengard P, Poo MM. Calcitonin gene-related peptide potentiates synaptic responses at developing neuromuscular junction. *Nature* 1993; 363: 76-79.

101. Dumoulin FL, Raivich G, Haas CA, Lazar P, Reddington M, Streit WJ *et al.* Calcitonin gene-related peptide and peripheral nerve regeneration. *Ann NY Acad Sci* 1992; 657: 351-360.

102. Sala C, Andreose JS, Fumagalli G, Lomo T. Calcitonin gene-related peptide: possible role in formation and maintenance of neuromuscular junctions. *J Neurosci* 1995; 15: 520-528.

103. Madison RD, Archibald SJ. Point sources of Schwann cells result in growth into a nerve entubulation repair site in the absence of axons: effects of freeze-thawing. *Exp Neural* 1994; 128: 266-275.

104. Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L *et al.* Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci USA* 1994; 91: 3739-3743.

105. Donnerer J, Schuligoi R, Stein C, Amann R. Up-regulation, release and axonal transport of substance P and calcitonin gene-related peptide in adjuvant inflam mation and regulatory function of nerve growth factor. *Regul Pept 1993;* 46: 150-154.

106. Lindholm D, Heumann R, Meyer M, Thoenen H. Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. *Nature* 1987; 330: 658-659.

107. Funakoshi H, Frisen J, Barbany G, Timmusk T, Zachrisson O, Verge VM *et al.* Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J CellBiol* 1993; 123: 455-465.

lOS. Battegay EJ. Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. *J Mol Med* 1995; 73: 333-346.

109. BhoraFY, DunkinBJ, Batzri S, Aly HM, Bass BL, Sidawy AN *et al.* Effect of growth factors on cell proliferation and epithelialization in human skin. *JSurgRes* 1995; 59: 236-244.

110. Fajardo LF, Prionas SD, Kwan HH, Kowalski J, Allison AC. Transforming growth factor-beta 1 induces angiogenesis in vivo with a threshold pattern. *Lab Invest* 1996; 74: 600-608.

lll. Ono I, Gunji H, Zhang JZ, Maruyama K, Kaneko F. Studies on cytokines related to wound healing in donor site wound fluid. *J Dermatol Sci* 1995; 10: 241-245.

112. Register B, Delree P, Leprince P, Martin D, Sadzot C, Malgrange B *et al.* Transforming growth factor-beta as a neuroglial signal during peripheral nervous system response to injury. *J Neurosci Res* 1993; 34: 32^43.

113. Bates DJ, Mangelsdorf DC, Ridings JA. Multiple neurotrophic factors including NGF-like activity in nerve regeneration chamber fluids. *Neurochem Int* 1995; 26: 281-293.

IH. Laquerriere A, Peulve P, Jin O, Tiollier J, Tardy M, Vaudry H *et al.* Effect of basic fibroblast growth factor and alpha-melanocytic stimulating hormone on nerve regeneration through a collagen channel. *Microsurgery* 1994; 15: 203-210.

115. Laird JM, Mason GS, Thomas KA, Hargreaves RJ, Hill RG. Acidic fibroblast growth factor stimulates motor and sensory axon regeneration after sciatic nerve crush in the rat. *Neuroscience* 1995; 65: 209-216.

116. Walter MA, Toriumi DM, Patt BS, Bhattacharyya TK, O'Grady K, Caulfield JB *et al.* Fibroblast growth factor-induced motor end plate regeneration in atrophic muscle. *Arch Otolaryngol Head Neck Surg* 1996; 122: 425-430.

117. Edbladh M, Fex-S venningsen A, Ekstrom PA, Edstrom A. Insulin and IGF-II, but not IGF-I, stimulate the in vitro regeneration of adult frog sciatic sensory axons. *Brain Res* 1994; 641: 76-82.

118. Newman JP, Verity AN, Hawatmeh S, Fee Jr WE, Terris DJ. Ciliary neurotrophic factors enhances peripheral nerve regeneration. *Arch Otolaryngol Head Neck Surg* 1996; 122: 399-403.

119. Sahenk Z, Seharaseyon J, Mendell JR. CNTF potentiates peripheral nerve regeneration. *Brain Res* 1994; 655: 246-250.

120. Glazner GW, Morrison AE, Ishii DN. Elevated insulin-like growth factor (IGF) gene expression in sciatic nerves during IGF-supported nerve regeneration. *Brain Res Mol Brain Res* 1994; 25: 265-272.

121. Scherer SS, Kamholz J, Jakowlew SB. Axons modulate the expression of transforming growth factor-betas in Schwann cells. *Glia* 1993; 8: 265-276.

122. Toma JG, Pareek S, Barker P, Mathew TC, Murphy RA, Acheson A *et al.* Spatiotemporal increases in epidermal growth factor receptors following peripheral nerve injury. *J Neurosci* 1992; 12: 2504-2515.

123. Nukada H. Post-traumatic endoneurial neovascularization

and nerve regeneration: a morphometric study. *Brain Res* 1988; 449: 89-96. ;. .

124. Wu L, Mustoe TA. Effect of ischemia on growth factor enhancement of incisional wound healing. *Surgery* 1995; 117: 570-576.

125.Ferrara N, Houck K, Jakeman L, LeungDW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 1992; 13: 18-32.

126. Giannini C, Dyck PJ. The fate of Schwann cell basement membranes in permanently transected nerves. *J Neuropathol Exp Neural* 1990; 49: 550-563.

127. Roytta M, Salonen V. Long-term endoneurial changes after nerve transection. *Acta Neuropathol* 1988; 76: 35-45.

128. Siironen J, Vuorinen V, Taskinen HS, RoyttaM. Axonal regeneration into chronically denervated distal stump. 2. Active expression of type I collagen mRNA in epineurium. *Acta Neuropathol* 1995; 89: 219-226.

129. Vuorinen V, Siironen J, Roytta M. Axonal regeneration into chronically denervated distal stump. 1. Electron microscope studies. *Acta Neuropathol* 1995; 89: 209-218.

130. Fu S Y, Gordon T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci* 1995; 15: 3886-3895.

131. Bredt DS, Snyder SH. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *ProcNatlAcadSci USA* 1990; 87: 682-685.

132. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* 1994; 63: 175-195.

133. Dawson VL, Dawson TM. Physiological and toxicologi-cal actions of nitric oxide in the central nervous system. *Adv Pharmacol* 1995; 34: 323-342.

134. Verge VM, Xu Z, Xu XJ, Wiesenfeld-Hallin Z, Hokfelt T. Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: *in situ* hybridization and functional studies. *Proc Natl Acad Sci USA* 1992; 89: 11617-11621.

135. Wu W, Liuzzi FJ, Schinco FP, Depto AS, Li Y, Mong JA *et al.* Neuronal nitric oxide synthase is induced in spinal neurons by traumatic injury. *Neuroscience* 1994; 61: 719-726.

136. Gross SS, Stuehr D J, Aisaka K, Jaffe EA, Levi R, Griffith OW. Macrophage and endothelial cell nitric oxide synthesis: cell-type selective inhibition by NG-amino-argi-nine, NG-nitroarginine andNG-methylarginine. *Biochem Biophys Res Commun* 1990; 170: 96-103.

137. Gold R, Zielasek J, Kiefer R, Toyka KV, Hartung HP. Secretion of nitrite by Schwann cells and its effect on T-cell activation in vitro. *Cell Immunol* 1996; 168: 69-77.

138.De Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris Jr SM *et al.* Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc Natl Acad Sci USA* 1996; 93: 1054-1059.

139. Levy D, Zochodne DW. Evidence for peripheral nitric oxide activity in a rat model of chronic neuropathic pain. *Soc Neurosci Abst* 1996; 342: 13.

140. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33: 87-107.

141.Thomas DA, Ren K, Besse D, Ruda MA, Dubner R. Application of nitric oxide synthase inhibitor, N omega-nitro-L-arginine methyl ester, on injured nerve attenuates neuropathy-induced thermal hyperalgesia in rats. *Neurosci Lett* 1996; 210: 124-126.

142. Kindgen-Milles D, Arndt JO. Nitric oxide as a chemical link in the generation of pain from veins in humans. *Pain* 1996; 64: 139-142.

143.Hess DT, Patterson SI, Smith DS, Skene JH. Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* 1993; 366: 562-565.

144.Radi R, Beckman JS, Bush KM, Freeman BA. Peroxy-nitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *JBiol Chem* 1991; 266: 4244-4250.

145. Zochodne DW, Misra M, Cheng C, Sun H. Inhibition of nitric oxide synthase enhances peripheral nerve regeneration in mice. *Neurosci Lett* 1997; 228: 71-74.

146. Devor M. Neuropathic pain and injured nerve: periphe-

ral mechanisms. *Br Med Bull* 1991; 47: 619-630.

147.Zimmerman M, Koschorke G-M. Chemosensitivity of nerve sprouts in experimental neuroma of cutaneous nerves of the cat. In: Schmidt RF, Schaible H-G, Vahle-Hinz C, editors. *Fine afferent nerve fibers and pain.* Weinheim, VCH, 1987; 106-113.

148.Nystrom B, Hagbarth KE. Microelectrode recordings from transected nerves in amputees with phantom limb pain. *Neurosci Lett* 1981; 27: 211-216.

149. Wall PD, Gutnick M. Properties of afferent nerve impulses originating from a neuroma. *Nature* 1974; 248: 740-743.

150. Wall PD, Gutnick M. Ongoing activity in peripheral nerves: the physiology and pharmacology of impulses originating from a neuroma. *Exp Neurol* 1974; 43: 580-593.

151. Amir R, Devor M. Chemically mediated cross-excitation in rat dorsal root ganglia. *J Neurosci* 1996; 16: 4733-4741.

152. Choi Y, Raja SN, Moore LC, Tobin JR. Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. *J Neurol Sci* 1996; 138: 14-20.

153.Munglani R, Harrison SM, Smith GD, Bountra C, Birch PJ, Elliot PJ *et al.* Neuropeptide changes persist in spinal cord despite resolving hyperalgesia in a rat model of mononeuropathy. *Brain Res* 1996; 743: 102-108.

154. Woolf CJ, ShortlandP, Reynolds M, Ridings J, Doubell T, Coggeshall RE. Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. *J Comp Neurol* 1995; 360: 121-134.

155. Devor M, Govrin-Lippmann R, Angelides K. Na^+ channel immunolocalization in peripheral mammalian axons and changes following nerve injury and neuroma formation. *J Neurosci* 1993; 13: 1976-1992.

156. England JD, Gamboni F, Ferguson MA, Levinson SR. Sodium channels accumulate at the tips of injured axons. *Muscle Nerve* 1994; 17: 593-598.

157. Lax H, Zochodne DW. "Causalgic" median mononeuropathies: segmental rubror and edema. *Muscle Nerve* 1995; 18: 245-247.

158.McLachlan EM, Jang W, Devor M, Michaelis M. Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. *Nature* 1993; 363: 543-546.

159. Schaafsma L, Sun H, Zochodne DW. Exogenous opioids influence the microcirculation of injured peripheral nerves. *Am J Physiol* 1997; 272: H76-H82.